Plant Cell Death System

The present invention relates to a plant cell death system, and in particular to transgenic plants which harbour within their genome a chimaeric gene which when expressed produces a cytotoxic protein.

One of the means open to plant breeders attempting to produce new cultivars is the production of hybrids between existing cultivars containing desirable traits. Hybrids are generally superior in a variety of characteristics to either parent, a phenomenon known as hybrid vigour. Such hybrid crosses may be performed by manual cross pollination, a tedious and time consuming procedure.

During the production of such hybrid crosses the prevention of self pollination is vital. To achieve this, the female parent may be emasculated by hand, e.g. in the production of hybrid corn by de-tasseling. However, the large scale emasculation of species with hermaphrodite flowers is economically unfeasible. Female parent lines (male sterile) may also be generated by genetic male sterility, a known trait in many plants, usually being recessive and monogenic. The problem with this approach is that it is difficult to obtain pure lines of male sterile parents for every cross. The most widely used system of producing male sterility for use in

hybrid production is cytoplasmic male sterility (cms). In this case cytoplasmic factors are responsible for pollen abortion. In crops where cms has been identified in the germplasm it has been used extensively, e.g. maize, sunflower. There are several disadvantages of the system: male sterile cytoplasm may be associated with other undesirable characteristics, e.g. T-cytoplasm in maize and susceptibility to *Helminthosporium maydis*; its application requires isogenic maintainer male fertile lines to propagate the male parent; and it is limited to species in which a cytoplasmic source of sterility is available.

Another advantage of a male sterility system would be the production of pollen-free plants. This would be desirable in a number of ornamental flower varieties, and would also have application in the containment of genetic traits by the prevention of outcrossing.

A further desirable property of a sterility system is that female sterile plants could be produced such that fruit development would occur in the absence of seed set. Seedless fruit varieties would be advantageous for processing e.g., tomatoes, and also desirable to the consumer, e.g. melon. Seedless varieties are available and there are established breeding programmes, but the development of seedless fruit has been limited by the availability of the appropriate germplasm in many species.

In cases where a genetic source of sterility is not available or is otherwise unfeasible, a genetic modification approach could provide sterility by providing a cell death system whereby necrosis occurs in specific cells in the reproductive tissues.

WO 89/10396 discloses a plant cell death system wherein a chimaeric gene is introduced into a plant, which chimaeric gene comprises an anther specific promoter attached to a RNAse protein or polypeptide which, when expressed, causes disruption of cell metabolism. Thus, expression of the chimaeric gene results in necrosis of the anther cells and results in male sterility in the plants.

A similar cell death system whereby the target site may be the ovule of the plant, could be used to provide female sterility in plants.

WO 93/18170 and WO 92/04453 disclose plant cell death systems which are specific to controlling nematode infection. In WO 93/18170 and WO 92/04453 a gene comprising a coding sequence, which coding sequence encodes for a product which is disruptive of nematode attack is introduced into a host plant species. The gene further comprises a promoter region, which promoter region controls the expression of the coding sequence such that expression occurs upon nematode attack and substantially specifically within or adjacent to the nematode feeding site cells. In order to disrupt nematode attack, the

product may be either inimical to the plant cells which differentiate into nematode feeding site cells or cells adjacent thereto, or inimical to the nematodes directly.

Economically important plant parasitic nematodes include cyst nematodes, such as potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*), soybean cyst nematode (*Heterodera glycines*), beet cyst nematode (*Heterodera schachtii*) and cereal cyst nematode (*Heterodera avenae*), and root knot nematodes, such as *Meloidogyne* spp. Such plant parasitic nematodes are major pathogens of many crops world-wide, for example vegetables, food legumes, tomato, water melon, grape, peanut, tobacco and cotton.

Chemical control, cultural practices and the use of resistant plant varieties are the chief approaches to nematode control which are currently available and they are often used in an integrated manner against plant parasitic nematodes. There is a requirement for improvement in nematode control because these current approaches offer inadequate crop protection. Nematicides are of questionable environmental status and they are not always efficacious. Cultural control imposes hidden losses on growers in several ways. The wide host range of root knot nematodes limits the availability of economically satisfactory non-host crops. Effective resistant cultivars are frequently unavailable and those that the grower can use are sometimes out-performed by susceptible cultivars

at low nematode densities. Also resistance may be lost in the high soil temperatures that occur in tropical and sub-tropical environments.

Other applications of plant cell death systems can be envisioned. For example the target site may be specific parts of the flower, thereby altering the morphology of the flower. Alternatively the target site may be lateral roots, thorns or stinging hairs. Abscission of leaf or fruit might be achieved by the targeting of the abscission zone of the leaf or the fruit. Facilitating the release of seeds from plants, by targeting the funicle might be achievable. By targeting other organs such as trichomes, which trichomes are typically glandular, the production of chemical substances by these organs can be cessated or prevented. Another application might be the inducible abscission of roots, leaves, flowers, or fruit at the end of the growing season.

In NZ 260511 a plant cell death system is proposed with increased tissue specificity. This system comprises the expression of a cytotoxic molecule (under the control of a first promoter, which first promoter causes expression in specific target cells and at one or more other sites in the plant), in conjunction with a protective molecule (under the control of a second promoter, which second promoter causes expression in all of the sites where the first promoter is active except the specific target cells). Examples of suitable

cytotoxic and protective molecules are proteases and protease inhibitors, respectively, or nucleases and nuclease inhibitors, respectively. WO 93/10251 discloses the use of a cytotoxic ribonuclease molecule Barnase together with the protective, inhibitor molecule Barstar.

WO 98/44138 discloses a method for improving the specificity of gene expression by targeting a specific expression site of a target gene. There is thus provided a chimaeric gene, which chimaeric gene comprises a promoter which expresses in more than one region of the organism to be affected. The promoter is linked to an agent which affects the functioning of an endogenous gene in the plant which is also expressed in more than one region of the plant. The promoter and agent are selected so that there is an overlap in their expression sites at one or more desired locations. This overlap site(s) gives increased specificity and targeting of gene expression.

Ribosome-inactivating proteins (RIPs) are a group of toxic plant proteins that catalytically inactivate eukaryotic ribosomes (Stirpe and Barbieri 1986). RIPs function as *N*-glycosidases to remove a specific adenine in a conserved loop of the large rRNA, and thereby prevent binding of Elongation Factor 2, thus blocking cellular protein synthesis.

Three forms of RIPs have been described. Type 1 RIPs such as pokeweed antiviral protein (PAP) and barley translation

inhibitor are each comprised of a single polypeptide chain, each with an approximate M_r value of 30,000. Type 2 RIPs such as ricin, abrin and modeccin each comprise two polypeptide chains. One polypeptide with RIP activity (A-chain) is linked by a disulphide bond to a galactose-binding lectin (B-chain; Stirpe *et al* 1978). The M_r value of each Type 2 RIP is approximately 60,000. Type 3 RIPs such as maize RIP comprise a single polypeptide chain which subsequently undergoes proteolytic cleavage to release two active peptide domains.

Pokeweed (*Phytolacca americana*) produces three distinct antiviral proteins, namely PAP', PAPII and PAP-S that appear in spring leaves, summer leaves and seeds, respectively. Amino acid similarities between these three proteins have been observed. As used herein 'PAP' covers all three of these antiviral proteins. PAP' or PAPII are stored in the cell wall matrix of leaf mesophyll cells and may be isolated by aqueous extraction of macerated leaf tissue (Ready *et al*, 1986). It has been found that their exogenous application to the surface of plant leaves can confer protection against infection by a range of viruses in several different host plants (Chen *et al*, 1991; Lodge *et al*, 1993).

US 6 015 940 discloses the preparation of a cDNA clone of PAP' prepared from spring leaves of pokeweed, and the use thereof under the control of a constitutive promoter (either cauliflower mosaic virus 35S promoter or the figwort mosaic

virus 35S promoter) in the production of transgenic tobacco and potato plants resistant to infection by the viruses PVX and PVY. A negative feature of the resistant phenotype was that plants which expressed PAP' at levels above 10ng/mg protein exhibited mottled leaves and stunted growth. The plants that accumulated the highest levels of PAP' were sterile.

Transgenic plants containing the summer leaf form of PAP, PAP-II, have been described in WO 99/60843. A number of full length and truncated PAP-II gene sequences were screened in order to identify those variant PAP-II proteins which retained antiviral activity but exhibited no phytotoxicity. Transgenic plants exhibited both antiviral and antifungal activity.

The PAP gene is expressed *in vivo* in leaves initially to produce an inactive Pro-PAP protein. It is known that following translation, the Pro-PAP' protein molecule is targeted to the cell wall. At some stage during this process the N- and C-terminal extensions of the Pro-PAP' molecule are cleaved to produce an activated PAP' molecule (mature PAP'). In the case of PAP-S (expressed in seeds) the cellular localisation is not known. However, the N-terminal processed region of PAP-S appears to have properties similar to signal sequences for targeting.

The structure of the mature PAP-S protein, i.e. with N- and C-terminal extensions removed, may be described in terms

of two separate domains, corresponding to the two domains of Type 3 RIPs, or the two polypeptides of Type 2 RIPs, i.e. the ribosome binding domain and the catalytic domain. Described herein are recombinant PAP-S molecules comprising separately the PAP-S α sequence or the PAP-S β sequence. On the basis of structural predictions, PAP-S α contains the RNA recognition motif and ribosome binding domain regions, whilst PAP-S β contains the critical catalytic residue site. The present invention demonstrates that, surprisingly, expression of either the PAP-S α protein or the PAP-S β protein alone results in a significant inhibition of ribosome activity.

The present invention provides a method of inducing a necrotic effect in specific cells of a plant, wherein a plant is transformed with a chimaeric gene, the coding sequence of said gene coding for a mature pokeweed antiviral protein or part thereof, said gene comprising a promoter which acts in response to the application of a specific stimulus to said plant, so that said mature pokeweed antiviral protein or part thereof is expressed in specific cells of said plant.

The present invention further provides a plant transformed with a chimaeric gene, the coding sequence of said gene coding for a mature pokeweed antiviral protein or part thereof, said gene comprising a promoter which acts in response to the application of a specific stimulus to said

plant, so that said mature pokeweed antiviral protein or part thereof is expressed in specific cells of said plant.

The present invention yet further provides a recombinant plant cell transformed with a chimaeric gene, the coding sequence of said gene coding for a mature pokeweed antiviral protein or part thereof, said gene comprising a promoter which acts in response to the application of a specific stimulus to said plant, so that said mature pokeweed antiviral protein or part thereof is expressed in specific cells of said plant.

The present invention also provides a DNA isolate comprising a chimaeric gene, the coding sequence of said gene coding for a mature pokeweed antiviral protein or part thereof, said gene comprising a promoter which acts in response to the application of a specific stimulus to said plant, so that said mature pokeweed antiviral protein or part thereof is expressed in specific cells of said plant.

The present invention further provides a biologically functional expression vehicle containing a chimaeric gene, the coding sequence of said gene coding for a mature pokeweed antiviral protein or part thereof, said gene comprising a promoter which acts in response to the application of a specific stimulus to said plant, so that said mature pokeweed antiviral protein or part thereof is expressed in specific cells of said plant.

As used herein the term 'part' means a part of a gene encoding for a pokeweed antiviral protein, which part is active in inhibiting protein synthesis.

The chimaeric gene preferably encodes the mature PAP-S protein, the nucleotide sequence being given in SEQ. ID. No. 3 and the amino acid sequence being given in SEQ. ID. No. 4, or PAP-S α, the nucleotide sequence being given in SEQ. ID. No. 5 and the amino acid sequence being given in SEQ. ID. No. 6, or PAP-S β, the nucleotide sequence being given in SEQ. ID. No. 7 and the amino acid sequence being given in SEQ. ID. No. 8, or mature PAP' or a variant thereof as described in US Patent No. 6,015,940 (i.e. nucleotides 290-1076 of SEQ. ID. *Nos.* 30 and 31), or mature PAPII as described in International Patent Application, Publication No. WO 99/60843 (i.e. nucleotides 75-903 of SEQ. ID. No. 32).

Depending on the homology of the nucleotide sequences required, different conditions of stringencies may be used in the hybridisation procedure used to screen for similar sequences. By way of example and not limitation, hybridisation procedures using conditions of high stringency are as follows: hybridisation to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley and Sons, Inc.,

New York, at p. 2.10.3). Other conditions of high stringency which may be used are well known in the art. Hybridisation procedures using conditions of moderate stringency that may be used are as follows: hybridisation to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al*, 1989, *supra*). Other conditions of moderate stringency which may be used are well-known in the art. Other solutions such as Standard Saline Citrate (SSC) or (Saline Sodium Phosphate EDTA) (SSPE) can be used in the hybridisation procedures.

Suitable homologous sequences are sequences that are at least 70%, preferably 80% and even more preferably 90% or 95% homologous with each sequence listed herein, which such homolgous sequences retain the required enzymatic activity.

The suitable sequences may also be variants thereof. Variant in relation to the present invention may mean any substitution of, variation of, modification of, replacement of or deletion of or the addition of one or more nucleic acid(s)/amino acids from or to the sequence, providing the resulting sequence expresses or exhibits the required enzymatic activity. A derivative or mutation may also be suitable in the invention. A derivative has some modifications, usually chemical, compared with the naturally-occurring polypeptide expressed by the nucleic acid.

The present invention provides in a second aspect a method of inducing a necrotic effect in specific cells of a plant, wherein a plant is transformed with two chimaeric genes, a coding sequence of one of said genes coding for an inactivated pokeweed antiviral protein, and a coding sequence of the other of said genes coding for a second molecule, which molecule is an activator of the inactivated pokeweed antiviral protein, each of said two genes comprising a promoter, which promoters act conjointly in response to the application of a specific stimulus to said plant so that said inactivated pokeweed antiviral protein is activated in specific cells of a plant.

The present invention further provides a plant transformed with two chimaeric genes, a coding sequence of one of said genes coding for an inactivated pokeweed antiviral protein, and a coding sequence of the other of said genes coding for a second molecule, which molecule is an activator of the inactivated pokeweed antiviral protein, each of said two genes comprising a promoter, which promoters act conjointly in response to the application of a specific stimulus to said plant so that said inactivated pokeweed antiviral protein is activated in specific cells of a plant.

The present invention yet further provides a recombinant plant cell, a DNA isolate of two chimaeric genes, and a biologically functional expression vehicle containing two

chimaeric genes, each chimaeric gene being in accordance with the second aspect of the invention hereof.

As used herein the term 'inactivated pokeweed antiviral protein' is a mature pokeweed antiviral protein, such as PAP-S, for example, operably linked to a heterologous or homologous N- or C-terminal blocking sequence.

The chimaeric gene preferably encodes the mature PAP-S protein, the nucleotide sequence being given in SEQ. ID. No. 3 and the amino acid sequence being given in SEQ. ID. No. 4, or PAP-S α, the nucleotide sequence being given in SEQ. ID. No. 5 and the amino acid sequence being given in SEQ. ID. No. 6, or PAP-S β, the nucleotide sequence being given in SEQ. ID. No. 7 and the amino acid sequence being given in SEQ. ID. No. 8, or mature PAP' or a variant thereof as described in US Patent No. 6,015,940 (i.e. nucleotides 290-1076 of SEQ. ID. *Nos.* 30 and 31), or mature PAPII as described in International Patent Application, Publication No. WO 99/60843 (i.e. nucleotides 75-903 of SEQ. ID. No. 32), each being linked to a heterologous or homologous N- or C-terminal blocking sequence as described above to produce an inactive pokeweed antiviral protein.

The terminal blocking sequence is advantageously a sequence that may have an additional necrotic effect on the cell or pathogen.

Preferably the terminal blocking sequence is the oryzacystatin sequence (Δ86 cystatin sequence) known herein as

SEQ. ID. No. 17. The Δ86 cystatin sequence blocks the action of natural proteases thus preventing natural expression of the PAP coding sequence. Cystatins are small (~100aa) protein inhibitors of cysteine proteases found in many plants [Ryan, 1990; Richardson, 1991] and a large number of genes (>60) have been sequenced. Phytocystatins represent a distinct class from the type I and II cystatins [Kondo *et al.* 1991]. Oryzacystatin I (OC-I) from rice seed [Abe *et al.*, 1987] is an efficient inhibitor, though significantly less potent than the animal cystatins, such as chicken egg white cystatin (CEWC). Recombinant variants of OC-1 produced via protein engineering have been described by Uwrin *et al.* (1995), and the deletion mutant Δ-Asp86 (OC-IΔD86) exhibited increased inhibitory activity.

Alternatively, the terminal blocking sequence is merely a mature PAP inactivating molecule. Advantageously the terminal blocking sequence is a native Pro-PAP terminal sequence from any Pro-PAP molecule.

The blocking sequence may be located at the N-terminal or the C-terminal end of the mature PAP sequence.

Preferably the blocking sequence is a C-terminal blocking sequence.

In order to allow for re-activation of the blocked mature PAP sequence a specific cleavage site is also required in the inactivated pokeweed antiviral protein and is located between

the mature pokeweed antiviral protein sequence and the blocking sequence. The cleavage site is preferably located between the mature pokeweed antiviral protein and the blocking sequence.

Preferably the specific cleavage site is the Tobacco Etch Virus (TEV) NIa Protease cleavage site (Carrington and Dougherty, 1998), known herein as SEQ. ID. NO. 28 and SEQ. ID. No. 29. The recognition site for this protease is the heptapeptide Glu-Xaa-Xaa-Tyr-Xaa-Gln-Gly or the heptapeptide Glu-Xaa-Xaa-Tyr-Xaa-Gln-Ser with cleavage occurring between Gln and Gly (or Ser).

The coding sequence for the second molecule is preferably a protease which cleaves the specific cleavage site. When the specific cleavage site is TEV PCS then the protease is suitably TEV NIa Protease. Other specific cleavage sites and proteases will be readily available to the skilled person. For example, the site specific protease Enterokinase could be used to cleave an appropriate cleavage site linked to the mature pokeweed antiviral protein.

The said stimulus in respect of the subject invention as applied to a plant may be constituted by a pathogenic attack. Alternatively the stimulus may be chemically induced or could be induced as a result of the natural development of the plant.

As will be realised by those skilled in the art, the first and second aspects of the subject invention have widespread application throughout the plant kingdom for protection from attack by, for example, fungi, nematodes, bacteria and viruses, and for other purposes.

It is to be understood that, as used herein, the term "necrotic effect" embraces the concept of substantial impairment of metabolism such that the objective, e.g. disease protection, of employing the subject invention is attained.

Preferably, the promoters of this second aspect of the present invention (the two component system) have an overlapping expression zone(s). The overlapping expression characteristics of the two promoters and the respective responses thereof to the said stimulus are such as to effect the direction of the expression of the inactivated pokeweed antiviral protein and the activator molecule therefor so that a lethal or detrimental effect is produced in, and only in, the specific cells.

Alternatively, the promoters may have the same expression sites or the expressed proteins may otherwise accumulate at the target site.

In accordance with a third aspect of the invention, the present invention provides a method of inducing a necrotic effect in specific cells of a plant, wherein the plant is transformed with a chimaeric gene, the coding sequence of said

gene coding for a precusor PAP molecule or a C-terminal deletion thereof, said gene comprising a promoter which acts in response to the application of a specific stimulus to the plant, so that the protein expressed by the coding sequence is expressed in specific cells of said plant, said promoter being appropriately selected to provide one of the following effects: nematode infection disruption, sterility, changes in flower morphology, abscission, seed release or trichome development.

The present invention further provides a plant transformed in accordance with the method of the third aspect of the invention. The present invention even further provides a recombinant plant cell, a DNA isolate of a chimaeric gene, and a biologically functional expression vehicle each containing a chimaeric gene as described in accordance with the third aspect of the invention.

In respect of the third aspect the coding sequence preferably encodes one of the following list: the Pro-PAP-S, the nucleotide sequence being given in SEQ. ID. No. 1 and the amino acid sequence being given in SEQ. ID. No. 2, or PAP', or a variant thereof as described in US Patent No. 6,015,940 known herein as SEQ. ID. *Nos.* 30 and 31, or PAPII as described in International Patent Application, Publication No. WO 99/60843 and known herein as SEQ. ID. No. 32.

The selection of suitable promoters will depend upon the specific cells. If protection is sought against nematode attack the specific cells may be the nematode feeding site cells. In which case, in the second aspect of the invention, suitable promoters would be the KNT1 promoter acting conjointly with the TobRB7 promoter. In the first and third aspects of the invention these promoters may be used individually. The isolation of the KNT1 promoter is taught in NZ Patent No. 260511 and is further recited below and the isolation of the TobRB7 promoter is taught in International Patent Application WO 94/17194. The subject matter thereof relating to such isolation is incorporated herein by reference. Further suitable promoters are the Lemmi promoters, the isolation of which is disclosed in International Patent Application, Publication No. WO 92/21757.

By way of example, in the second aspect of the invention the inactivated pokeweed antiviral protein coding sequence may be under the control of the TobRB7 promoter whilst the activator molecule coding sequence may be under the control of the KNT1 promoter. The promoter KNT1 is expressed in nematode feeding site cells, root tips and to a lesser extent in other meristems, whereas the TobRB7 promoter is expressed in roots and giant cells (Conkling *et al* 1990). In fact studies have shown the TobRB7 promoter to be expressed in the body of the root but not the root tips. Thus the overlapping expression

zone of the KNT1 and TobRB7 promoters is in the giant cells (nematode feeding site cells) of the plant.

As an alternative example the specific cells may be anther cells to cause male sterility in plants. Examples of suitable promoters are disclosed in Twell *et al* (1991) and Mariani *et al* (1990).

In accordance with a first embodiment of the present invention, the target site may be a nematode feeding site. When it is the case that the target site is a nematode feeding site, the promoter selected is one which is induced at and/or adjacent to the nematode feeding site. Such a promoter is preferably induced upon nematode infection of the plant. An example of a suitable promoter is the KNT1 promoter. Other suitable promoters include the TobRB7 promoter and the Lemmi promoters.

The nematode feeding site may be comprised of, for example, plant cells at the local site of infection which later redifferentiate to form a syncytium (in the case of cyst nematodes) or the giant cells and/or the accompanying hypertrophic cells (in the case of root knot nematodes), and/or one or more of the syncytium cells, the giant cells and the accompanying hypertrophic cells.

By targeting the nematode feeding site a nematode resistant plant may be obtained. By the term "nematode resistant plant" it is meant a plant which upon infection by

plant parasitic nematodes is capable of preventing, slowing or otherwise adversely affecting the growth and development of nematodes that attack the plant, thereby preventing economically significant densities of plant parasitic nematodes from building up during a single crop growing period. That is to say that the nematodes may, for example, die or the nematodes' life cycle may be slowed resulting in a delay in the time taken to reach maturity and hence produce eggs, or the mature female nematodes may be of reduced size and thus have a lower egg-laying capacity as egg laying only commences after female nematodes have reached a critical, minimum size.

The present invention is applicable to, but in no way limited to, use with the following nematode species: *Globodera* spp., *Heterodera* spp. and *Meloidogyne spp*.

In accordance with a second embodiment of the present invention, instead of nematode resistance the method is directed to effecting male sterility in plants. For example, the target site may be one or more of a plant's pollen, anther or tapetum. When it is the case that the target site is tapetum for example, the promoter selected is one that is induced in and/or adjacent to the tapetum. An example of a suitable tapetum promoter is the tobacco TA29 promoter as disclosed in Mariani *et al* (1990). Anther specific promoters are disclosed in Twell *et al* (1991).

In accordance with a third embodiment of the present invention, the method is directed to effecting female sterility in plants. For example, the target site may be the ovule of the plant. That is to say, the promoter selected is one that is induced in and/or adjacent to the ovule. An example of a suitable promoter is the AGL15 promoter as disclosed in Perry *et al*, 1996.

According to a fourth embodiment of the present invention, the morphology of the flower of a plant is manipulated. For example, the target site may be specific parts of the flower, the aim being that when these specific parts of the flower do not develop the morphology of the flower is changed. In that instance, the promoter selected is one that is induced in and/or adjacent to the sepal, carpel, petal, and/or stamen. Examples of suitable promoters are the found in the agamous, apetala3, globosa, pistillata and deficiens genes (Sieburth and Meyerowitz, 1997; Samach *et al*, 1997, and references therein).

In accordance with a fifth embodiment of the present invention, the method is used to assist in or promote leaf and/or fruit abscission in plants. For example, the target site may be the abscission zone of the leaf and/or the fruit. Thus, the promoter selected is one that is induced in and/or adjacent to such an abscission zone.

A sixth embodiment of the present invention is the targeting of trichomes, which trichomes are typically glandular. The promoter selected is one that is induced in and/or adjacent to the trichomes. By causing necrosis of the trichomes of the plant the production of chemical substances by the trichome can be cessated or prevented. A seventh embodiment of the present invention is the targeting of lateral roots, thorns or stinging hairs.

In accordance with an eighth embodiment of the present invention the method is directed to the control of virus infections. During virus infections there are a number of genes which are induced specifically, or substantially specifically, within the cells actually infected by the virus. The promoter selected is one that is induced in and/or adjacent to the cells infected by the virus

According to a ninth embodiment of the present invention the method is directed to facilitating the release of seeds from plants, by targeting the seeds. During seed development there are a number of genes which are induced specifically, or substantially specifically, within certain cells/parts of the seed.

In accordance with a tenth embodiment of the present invention a promoter that is externally inducible and that is induced in, for example, the roots of the plant is selected. Such a promoter could be used to effect root abscission at the

end of a growing season. Comparable promoters induced in, for example, leaf petioles, pedicels or peduncles, could be used to effect abscission of leaves, flowers, or fruit at the end of the growing season.

Suitably, each chimaeric gene further comprises a 3' untranslated, terminator sequence. The terminator sequence may be obtained from the plant, bacterial or viral genes. Suitable terminator sequences are the pea *rbcS* E9 terminator sequence, the *Nos* terminator sequence derived from the nopaline synthase gene of *Agrobacterium tumefaciens* and the 35S terminator sequence from cauliflower mosaic virus, for example. A person skilled in the art will be readily aware of other suitable terminator sequences. In addition, the chimaeric gene may optionally comprise transcriptional or translational enhancer sequences, such as those described in International Patent Application, Publication No. WO 97/20056, intracellular targeting sequences and introns for example, as well as nucleotide sequences operable to facilitate the transformation process and the stable expression of the chimaeric gene, such as T-DNA border regions, matrix attachment regions and excision/recombination sequences.

Techniques for transforming plants are well known within the art and include *Agrobacterium*-mediated transformation, for example. Typically, in *Agrobacterium*-mediated transformation a binary vector carrying a foreign DNA of interest, i.e. a

chimaeric gene, is transferred from an appropriate *Agrobacterium* strain to a target plant by the co-cultivation of the *Agrobacterium* with explants from the target plant. Transformed plant tissue is then regenerated on selection media, which selection media comprises a selectable marker and plant growth hormones.

Further suitable transformation methods include direct gene transfer into protoplasts using polyethylene glycol or electroporation techniques, particle bombardment, micro-injection and the use of silicon carbide fibres for example.

Suitable plant species which may be transformed in accordance with the present invention include, but are not limited to rice, wheat, maize, canola, potato, tobacco, sugar beet, soybean, tomato, peanut, cotton, vine, watermelon, papaya, vegetables and food legumes.

Suitably, in the second aspect of the invention, both chimaeric genes are introduced into the plant in a single expression cassette. Alternatively, each chimaeric gene is introduced into the plant in a separate expression cassette through sequential or simultaneous transformation with two transgene constructs. Such a two-component system may also be produced by the crossing of two plants each containing one individual component.

In order that the invention may be easily understood and readily carried into effect, reference will now be made, by

way of example, to the accompanying diagrammatic drawings, in which:

Figure 1 shows in schematic form promoter constructs for PAP expression in protoplasts;

Figure 2 shows the vector pDVM;

Figure 3 shows the plant transformation vector pATC;

Figure 4 shows in schematic form nematode inducible promoter constructs for PAP expression in transgenic plants;

Figure 5 shows the effect of PAP-S protein on tobacco ribosomes as measured by the GUS protein synthesis;

Figure 6 shows the effect of modified PAP-S proteins on tobacco ribosomes as measured by the GUS protein synthesis;

Figure 7 shows the effect of PAP-S α and PAP-S β on protoplasts;

Figure 8 shows in schematic form the PAP-S/Protease Cleavage Site (PCS)/Cystatin ΔD86 fusion construct, and the constructs in pBluescript vectors used in TnT experiments;

Figure 9 shows the effect of PAP proteins on translation of luciferase protein in rabbit reticulocyte lysates;

Figure 10 shows the effect of modified PAP-S/Cystatin protein on tobacco ribosomes as measured by the GUS protein synthesis;

Figure 11 shows in schematic form the nematode inducible promoter construct for regulated expression of PAP-S-PCS-Cystatin ΔD86 and TEV NIa protease in transgenic plants;

Figure 12 shows a resistance screen of potato plants transformed with Pro-PAP-S and infected with potato cyst nematode race 2/3 compared to a susceptible control (Hermes) and a commercial resistant cultivar (Sante);

Figure 13 shows a Root Knot Nematode resistance trial of tobacco plants containing the Pro-PAP-S construct (pATC05502) compared to tissue culture control plants (ncc). Sizes of infecting nematodes are recorded in eyepiece graticule units;

Figure 14 shows a schematic diagram of the production of PAP-S variants by PCR;

Figure 15 shows a comparison of mean cyst counts in potato plants transformed with genes containing Pro PAP-S, KNT1 antisense or Cowpea Trypsin Inhibitor (CpTI).

EXAMPLES

Cloning and Sequencing of Pokeweed Antiviral Protein (PAP-S) from Pokeweed Genomic DNA

The Pro-PAP-S sequence was isolated from pokeweed leaf DNA by polymerase chain reaction (PCR) using *Pfu* polymerase. The sequence was isolated in two segments, and which were then combined, in order to remove an *XbaI* site in the centre of the molecule. The 5′ portion of the molecule was amplified using primers PPS1BF and PSXDR (SEQ. ID. *Nos.* 9 and 16 respectively), and the 3′ portion using primers PSXDF and PPS2SR (SEQ. ID. *Nos.* 15 and 10 respectively). The two PCR

products were combined, and the overlapping DNA fragments extended with *Pfu* polymerase, and reamplified using primers PPS1BF and PPS2SR (SEQ. ID. *Nos.* 9 and 10 respectively). The PCR product was digested with restriction endonucleases *XbaI* and *SalI* and cloned into pBluescript to produce the clone pBS/Pro-PAP-S (Figure 14).

Using the pBS/Pro-PAP-S clone as template, the PAP-S sequence was amplified by PCR using primers PS1BF and PS2SR (SEQ. ID. *Nos.* 11 and 12 respectively) to eliminate the N- and C-terminal regions. A methionine start codon and a TAA stop codon were added via the PCR primers. The start and stop codons are shown in SEQ. ID. No. 3 but the enzyme cloning sites are not shown in SEQ. ID. No. 3. The added methionine residue is shown in SEQ. ID. No. 4. The PCR product was digested with restriction endonucleases *XbaI* and *SalI* and cloned into pBluescript to produce the clone pBS/PAP-S. Sequencing of the PAP-S clone revealed only one nucleotide change from the database sequence. Suitable primer combinations were used to remove either the N- or the C-terminal regions independently.

After comparison of PAP-S and Maize RIP proteins, nominal PAP-S α and PAP-S β polypeptide domains were defined based on the corresponding Maize RIP α,β and central domain regions (described in our co-pending application of even date). PAP-S α and PAP-S β polypeptide domains were amplified from PAP-S

clone plasmid DNA using PS1BF plus PS1SR (SEQ. ID. *Nos.* 11 and 14 respectively) and PS2BF plus PS2SR primer pairs respectively (SEQ. ID. *Nos.* 13 and 12 respectively). Methionine start codons and TAA stop codons were added via the PCR primers. The start and stop codons are shown in SEQ. ID. *Nos.* 5 and 7 but the enzyme cloning sites are not shown in SEQ. ID. *Nos.* 5 and 7. The added methionine residue is shown in SEQ. ID. *Nos.* 6 and 8. The amplified recombinant PAP-S α and PAP-S β sequences were cloned into separate vectors.

Isolation of a promoter that is induced at and/or adjacent to a target site

A method is hereby presented for the isolation of a promoter, which method is by way of an example. Alternative methods for the isolation of a suitable promoter for use in the present invention will be readily available to the skilled person, some of which methods are referenced above.

A method for the isolation of the KNT1 promoter is as follows:

Growth and Infection of Tobacco Plants

Seed of C319 tobacco were germinated on Fisons F1 compost under conditions as follows: light intensity of 4500 to 5000 lux; 16hr day/8hr night; temperature 20-25°C. After c. 3 weeks seedlings were gently washed in tap water to remove soil and transferred to pouches (Northrup-King), 2 plants per pouch,

and grown for a further week in a Conviron at 25°C with lighting as above. Roots were lifted from the back of the pouch and supported with Whatman GF/A glassfibre paper at their tips. Three-day-old nematodes (M.javanica) were then delivered to the tips of these roots in 10μl (50 nematodes) aliquots and a second piece of GF/A paper was placed on top to fully encapsulate the root tip. Following 24 hours post infection, the GF/A paper was removed to ensure synchronous infection. Following 3 days post infection the root knots were dissected out (leaving healthy root and root tip tissue behind) and frozen immediately in liquid nitrogen. Approximately 0.5-1.0g of infected root tissue was harvested from 80 inoculated plants.

Staining for visualisation of nematodes in infected roots

The quality of infection was established by determining the number of nematodes infecting per root tip. Roots were harvested from 3 day post infected plants and immersed for 90 seconds in lactophenol containing 0.1% Cotton Blue at 95°C. Following a 5 second rinse in water, the roots were placed in lactophenol at room temperature (RT) for 3-4 days to clear. Stained nematodes were visualised using light microscopy.

RNA isolation from healthy and infected root tissue

Root tissue was ground to a fine powder in a liquid nitrogen chilled pestle and mortar. Approximate 100mg aliquots were then transferred to similarly chilled microfuge tubes and 300μl of hot phenol extraction buffer (50% phenol, 50% extraction buffer: 0.1M lithium chloride, 0.1M Tris-HCl pH8.0 (RT), 10mM EDTA, 1% SDS) added, and incubated at 80°C for 5 minutes. An equal volume of chloroform was then added and the homogenate microfuged for 15 minutes at 4°C. The aqueous phase was then extracted with 600μl of phenol/chloroform and microfuged as above. The aqueous phase was again removed and the RNA precipitated with an equal volume of lithium chloride at 4°C overnight. The precipitate was pelleted by microfugation for 15 minutes at RT and washed in 70% ethanol. The pellet was lyophilised, resuspended in DEPC-treated water and assayed using a spectrophotometer. RNA quality was assessed by denaturing gel electrophoresis. (Adapted from Shirzadegan *et al*, 1991).

Subtractive cloning of infection specific cDNAs

Poly(A)$^+$ RNA (mRNA) was isolated from 200μg total RNA samples from healthy and infected C319 root tissue using magnetic oligo dT Dynabeads according to the manufacturer's instructions. First strand cDNA synthesis was performed in situ on the Dynabead-bound poly(A)$^+$ fraction from healthy

tissue to provide Driver DNA. First and second strand synthesis was performed in situ on the Dynabead-bound poly(A)$^+$ fraction from the infected tissue to provide Target DNA. All cDNA reactions were carried out using a cDNA synthesis kit according to the manufacturer's instructions (Pharmacia). Three oligonucleotides, SUB21 (5' CTCTTGCTTGAATTCGGACTA 3') known herein as SEQ. ID. No. 30, SUB25 (5' TAGTCCGAATTCAAGCAAGAGCACA 3') (sequences from Duguid & Dinauer, 1990) and known herein as SEQ. ID. No. 31 and LDT15 (5' GACAGAAGCGGATCCd$(T)_{15}$ 3') (O'Reilly, 1991) known herein as SEQ. ID. No. 32 were kinased with T4 polynucleotide kinase according to Maniatis *et al* (1982). SUB21 and SUB25 were then annealed to form a linker which was then ligated to the Target DNA with T4 DNA ligase according to King & Blakesley (1986). Subsequently the beads carrying the Target DNA were washed extensively with TE and the second strand of the cDNA eluted at 95°C in 5xSSC.

The RNA bound to the Dynabead-bound Driver DNA was removed by heat and the eluted Target DNA hybridised to the Driver DNA at 55°C in 5xSSC for 5 hours. Non-hybridising Target DNA was separated from the bead-bound Driver DNA at room temperature (RT) as per the manufacturer's instructions, following which, hybridising Target DNA was similarly separated from the bead-bound Driver DNA at 95°C. The RT eluted Target DNA was then added back to the Driver DNA and

the hybridisation repeated. This process was repeated until the amount of Target hybridising to the Driver no longer exceeded the amount that did not hybridise. DNA concentrations were established using DNA Dipstick (Invitrogen) in accordance with the manufacturer's instructions.

Aliquots of the final RT-eluted fraction were used in PCR amplification (Eckert and Kunkel, 1990) to generate double-stranded cDNA for cloning into a plasmid vector. Amplification of the target DNA was achieved using primers SUB21 and LDT15 according to the conditions described by Frohman *et al*, 1988. The PCR products were then ligated into SmaI digested pBluescript vector according to King and Blakesley (1986).

Screening of the subtractive library by Reverse Northern analysis

Recombinants were identified by colony PCR (Gussow and Clackson, 1989). The amplified inserts were Southern blotted in triplicate onto Pall Biodyne membranes and prescribed by the manufacturer. Prehybridisation and hybridisation were both carried out at 42°C in 5xSSPE, 0.05% BLOTTO, 50% formamide. Membranes were hybridised separately to cDNA probes (see below) from healthy and infected tissue and to a probe comprising amplified Target DNA from the final

subtraction. Clones showing a hybridisation signal to the infected cDNA probe only, or showing a hybridisation signal to the subtracted probe only were selected for further analysis.

cDNA probe generation

Samples of 10μg total RNA from healthy and infected tissue were treated with 2.5 units DNase1 at 37°C for 15 minutes. The DNase1 was then denatured at 95°C for 10 minutes before cDNA synthesis was performed using the manufacturer's protocol (Pharmacia). The RNA was then removed by the presence of 0.4M sodium hydroxide for 10 minutes at RT and the cDNA purified through a spun Sephacryl 400HR column. Yield and concentration was determined using DNA Dipsticks (Invitrogen). The cDNA was labelled, using c. 35ng/probe using the standard Pharmacia oligolabelling protocol.

Northern Blotting

To determine the expression profile of the clones selected from Reverse Northerns, they were used as probes in Northern analysis of either total or poly(A)$^+$ RNA from healthy and infected roots, stems, leaves, and flowers. Total RNA blots comprised 25μg RNA per lane, whilst poly(A)$^+$ RNA blots comprised 0.5-1.0μg RNA per lane. The RNA was electrophoresed on formaldehyde gels and blotted onto Pall Biodyne B membrane

as described by Fourney *et al* (1988). Probes were labelled and hybridised as above.

Southern Blotting

To determine whether selected cDNAs were of plant or nematode origin, tobacco C319 and M.javanica DNA were prepared as described by Gawel and Jarret (1991). Southern blots were prepared comprising 10μg EcoRI and HindIII digested DNA per lane. The blots were hybridised to oligolabelled probes as described above.

In Situ hybridisations

To determine the locality of expression of the cDNAs of interest at the feeding site, in situ hybridisations were performed. Tissue from infected and healthy roots were embedded in wax, sectioned, and hybridised to the probes as described by Jackson (1991).

Isolation of 5' termini of mRNAs

The 5' termini of the RNAs of interest were determined by using 5' RACE as described by Frohman *et al* (1988).

Isolation of promoter regions

The promoter regions of the genes of interest were isolated by vector-ligated PCR. 100ng samples of restriction

endonuclease digested C319 genomic DNA were ligated for 4 hours at RT (King and Blakesley, 1986) with 100ng samples of pBluescript (digested with a restriction endonuclease producing compatible termini). Typically enzymes used were EcoRI, BamHI, HindIII, BglII, XhoI, ClaI, SalI, KpnI, PstI, and SstI. PCR was then performed on the ligations using a vector primer such as the -40 Sequencing primer and a primer complementary to the 5' terminus of the mRNA. The PCR products were then cloned and sequenced. If necessary the process was repeated with a new primer complementary to the 5' terminus of the promoter fragment to ensure that the control sequences of the promoters were isolated.

Using the procedures described above, a gene, KNT1, was identified and isolated from tobacco plants. A KNT1 promoter fragment of approximately 0.8Kbp in length from the transcription start site, was isolated and inserted into the GUS reporter vector, pBI101 (Jefferson *et al*, 1987). The resulting construct, pBIN05101, was used to transform tobacco plants. Upon infection with M.javanica, strong GUS expression was observed in the nematode feeding site.

The KNT1 gene was shown to have homologues in species of plant other than tobacco. These include, but are not limited to Solanum tuberosum, Lycopersicon esculentum and Beta vulgaris. The KNT1 gene is also induced by both root knot and cyst nematode species.

The construct pBIN05101 was deposited by Advanced Technologies (Cambridge) Limited of 210 Cambridge Science Park, Cambridge CB4 0WA, England under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collections of Industrial, Food and Marine Bacteria (NCIMB), 23 St. Machar Street, Aberdeen, Scotland on 20 March 1997 under accession number NCIMB 40870. The vector contains the left and right borders of *Agrobacterium tumefaciens* C58 strain T-DNA. Between the borders are a multiple cloning site and a kanamycin resistance gene under the control of a plant promoter (*Nos*). External to the borders the vector contains a bacterial kanamycin resistance gene. The insert in the vector consists of a KNT1 promoter - glucuronidase coding sequence - *Nos* terminator.

Production of PAP-S constructs.

Constructs of PAP-S transgenes were made in vectors suitable for use in a transient expression protoplast system (Figure 1). The constructs were made in the high copy number vector pDE4 (Denecke *et al*, 1990), using a CaMV 35S constitutive promoter. The following constructs were made:

1. pDE4/Pro-PAP-S
2. pDE4/PAP-S ΔN
3. pDE4/PAP-S ΔC

4. pDE4/PAP-S

5. pDE4/PAP-S α

6. pDE4/PAP-S β

Constructs containing PAP-S effector components were also prepared in binary vectors with different promoters for plant transformation studies. The PAP-S sequences were first cloned into pDVM donor vectors (as shown in Figure 2) containing the nematode-inducible promoter KNT1 and the *Nos* terminator. The promoter-gene-terminator cassettes were then excised and cloned into the pATC binary vector (Figure 3) and introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

Constructs suitable for use in any of the other embodiments of the invention are similarly prepared using the same techniques described above and selecting appropriate targeting promoters.

Transient assay of PAP-S constructs in tobacco protoplasts

In this assay PAP-S mediated ribosome inactivation was detected by way of assessing GUS protein synthesis. Protoplasts were prepared from leaves of *in vitro*-maintained tobacco plants and were electroporated with the PAP-S constructs. The protein translation efficiency of ribosomes in the tobacco protoplasts was evaluated with a GUS reporter gene in a pDE4 construct under the control of a CaMV 35S promoter.

A GUS pDE4 construct was co-electroporated with each PAP-S construct. In order to obtain values for the optimum levels of GUS protein synthesis in protoplasts in competition with a second protein, a GUS-positive control was used, wherein a non-toxic BiP chaperone protein construct, pDE800 (Leborgna-Castel *et al*, 1999) was co-electroporated into tobacco protoplasts together with the GUS pDE4 construct. A GUS-negative control was also used, wherein an empty pDE4 vector was electroporated into tobacco protoplasts together with the non-toxic BiP chaperone protein construct.

(i) Mature PAP-S.

PAP-S constructs were co-electroporated with the GUS construct into tobacco protoplasts. The effect of PAP-S activity on ribosomes was assayed by measuring the levels of GUS activity after 24 hours of expression (Figure 5). The results indicate that PAP-S β protein inactivates tobacco ribosomes efficiently. Consequently only basal levels of GUS activity were observed in comparison with the GUS positive control.

(ii) Modified PAP-S constructs .

Different versions of PAP-S proteins were analysed in tobacco protoplasts (Figure 6).

Intact Pro-PAP-S was equally as effective as the mature PAP-S protein in inhibiting protein translation, supporting the hypothesis of its activation by endogenous proteases. However modifications to Pro-PAP-S sequence prevent this activation. The roles of the N and C terminal extensions of PAP-S protein appear to differ, in that prior deletion of the C-terminal region of Pro-PAP-S has little effect on subsequent ribosome inactivation, but an equivalent deletion of the N-terminal region of Pro-PAP-S produces a molecule which cannot be processed into an active form.

This provides a two component activation system based on a Δ-N Pro-PAP-S with a specific cleavage site introduced adjacent to the C-terminal region, or alternatively, or in addition, a Δ-C Pro-PAP-S with a sequence which blocks endogenous proteases but acts as a specific cleavage site introduced adjacent to the N-terminal region.

(iii) The separate PAP-S domains.

PAP-S α and PAP-S β polypeptide regions were also expressed either individually or in combination in tobacco protoplasts (Figure 7).

Surprisingly expression of either the PAP-S α protein or the PAP-S β protein alone resulted in a significant reduction in the GUS activity. On the basis of structural predictions,

PAP-S α contains the RNA recognition motif and ribosome binding domain regions, whilst PAP-S β contains the critical catalytic residue site. PAP-S α protein may be preventing protein translation by binding to ribosomes and preventing protein translation. The results imply that PAP-S α is adopting a correctly folded conformation and is capable of specific molecular recognition. PAP-S β contains the active site residue necessary for ribosome depurination but might not be expected to be capable of recognising and interacting with the ribosome. However, the results demonstrate that surprisingly PAP-S does have the ability to inhibit protein synthesis. This would imply that PAP-S β alone is adopting a correctly folded conformation and is capable of specific molecular recognition and catalytic cleavage. The ribosome inactivation achieved by PAP-S β alone is greater than that of PAP-S α.

Expression of both PAP-S α and PAP-S β simultaneously in protoplasts resulted in no further reduction of GUS activities in comparison with PAP-S β protein alone. This is unlike the situation observed with maize RIP α and RIP β domains where an enhancement is achieved, as described in our co-pending application of even date.

Construction of an inactive form of PAP-S by structural modification:

Inhibition of PAP action by preventing rRNA binding might be mediated by generating a fusion protein extending into the active site vicinity of PAP.

A suitable component selected for creating a fusion protein was the cystatin protein from rice (oryzacystatin), and a synthetic version of the gene was synthesised containing the codon usage pattern suitable for optimal expression in potato (SEQ. ID. No. 17). To ensure that the cystatin would occupy a position in the region of the PAP active site, 7 amino acids were deleted from the C-terminus of PAP. The fusion with the cystatin was created using a linker sequence encoding the cleavage site for NIa protease of Tobacco Etch Virus (TEV).

Primers as described below were designed to construct a fusion between PAP-S and rice cystatin ΔD86 with a TEV protease cleavage site (PCS) between them as a linker (Figure 8). The sequence encoding the fusion protein was amplified from cloned DNA in two PCR fragments; (i) PAP-S plus PCS was amplified using primers PS1BF and PCSPAPSR (SEQ. ID. *Nos.* 11 and 20), and (ii) Cystatin Δ86 with PCS was amplified using primers PCSDelta86F and SYNPOTDelta86SR (SEQ. ID. *Nos.* 21 and 22). The two PCR fragments were then used to amplify the full length fusion protein (PAPS-PCS-Cystatin Delta86) by overlap

extension PCR using PS1BF and SYNPOTDelta86SR primers (SEQ. ID. *Nos.* 11 and 22).

In this procedure, eight amino acids from the C terminal end of PAPS were removed and the TEV NIa protease cleavage site (seven amino acids) was incorporated, by means of primer PCSDelta86F (SEQ. ID. No. 21), encoding the heptapeptide: Glu Pro Val Tyr Phe Gln Gly. Then the Cystatin was added as a fusion protein at the C terminal end of the PAP-S-PCS sequence.

The fusion PCR product was cloned into pBluescript vector and was confirmed by sequence analysis.

Functional assay of inactivated PAP-S protein in an *in vitro* transcription/translation system

The constructs pBS/Pro PAP-S, pBS/PAP-S, and pBS/PAP-S/PCS/CystatinΔD86 were assessed in a rabbit reticulocyte lysate system. The TnT T7 quick coupled transcription and translation system (Promega Corporation) was used.

TnT reactions were incubated at 30°C for a total period of 60 minutes with different PAP constructs along with positive control luciferase construct. After initial incubation of PAP constructs in TnT reactions for 30 minutes, an equal quantity of luciferase template was added to all the reactions and continued for a further 30 minutes. Aliquots of different reactions were mixed with Luciferase Assay Reagent and the

luciferase activity was measured in a luminometer for 10 seconds (Figure 9).

The results of Pro PAP-S and PAP-S protein translation indicated that the proteins inactivated the translation by rabbit ribosomes as expected. The modified PAP fusion protein was relatively inactive in comparison with PAP. The translation of different PAP proteins was confirmed by incorporation of biotinylated lysyl tRNA as label during translation and detected with streptavidin conjugated alkaline phosphatase. The Pro PAP-S and PAP-S proteins translated were undetectable due to rapid ribosome inactivation whereas a relatively large amounts of modified PAP fusion protein was observed, indicating the relative inactivity of this PAP fusion protein towards rabbit ribosomes.

Cloning the TEV NIa Protease

The TEV NIa protease gene was amplified from a cDNA clone of TEV. A *Spe*I restriction site was removed for cloning purposes using the overlapping removal primers. The NIa protease was first amplified as two PCR fragments using primers NIAPROBF (SEQ. ID. No. 23) plus NIAPPROSR (SEQ. ID. No. 26) and NIAPROSDF (SEQ. ID. NO. 25) plus NIAPROSR (SEQ. ID. NO. 24) and a full length protease was then amplified by overlap extension PCR using primers NIAPROBF (SEQ. ID. No. 23) plus NIAPROSR (SEQ. ID. No. 24).

Transient protoplast assay of PAP-S fusion protein.

The effect of modified PAP-S/Cystatin on tobacco ribosomes was analysed in protoplasts, and the protein was shown to be have significantly reduced activity when compared to the unmodified protein (Figure 10). However, co-expression of TEV NIa protease together with the modified PAP-S/Cystatin protein resulted in an increase in ribosome inactivation. The separate expression of TEV NIa protease did not affect GUS protein synthesis.

Two component system for nematode-inducible expression of modified PAP-S

To test the effectiveness of the modified PAP-S protein and its activation with TEV NIa protease, a double construct was made using two nematode-inducible promoters, KNT1 (described above) and KNT2 (SEQ. ID. No. 27). KNT2 expresses in the root body (but not in the root tips) and giant cells of a nematode infected plant.

TEV NIa protease gene was amplified with *Pfu* DNA polymerase and the PCR product was cloned as an *XbaI* and *SalI* fragment into pBluescript vectors. The recombinant clone was sequenced to confirm its identity. PAP-S-PCS-Cystatin ΔD86 fusion and TEV NIa protease genes were cloned separately into pDVM donor vectors as individual constructs with KNT2 and KNT1

promoters respectively. The double construct (Figure 11) was made in a pDVM intermediate donor vector and recloned into a pATC binary plant transformation vector for generation of transgenic plants and nematode resistance trials.

Production of transgenic plants

Two constructs, pATC05501, containing the fully processed mature version PAP-S, and pATC05502, containing the precursor Pro-PAP-S (Figure 4) were introduced into tobacco and potato by the leaf disc co-cultivation method of Horsch *et al* (1985) using *Agrobacterium tumefaciens* strain LBA4404.

Attempts to transform tobacco with pATC05501 (mature PAP-S) failed to yield callus or shoots despite repeated experiments. Control constructs yielded transgenic shoots as expected showing that the effect was dependent on the mature PAP-S sequence.

In contrast, pATC05502 (Pro-PAP-S) gave rise to transgenic tobacco plants.

In the case of potatoes, there was no difficulty in recovering transgenic plants with either the mature PAP-S or the precursor Pro-PAP-S construct.

Nematode resistance screening of tobacco transformed with PAP-S constructs

Transgenic Pro-PAP-S tobacco plants were entered into a standard resistance trial with root knot nematodes *Meloidogyne javanica*. Transgenic and control tobacco plantlets were planted in a randomised blind experimental design into rootrainers fine potting compost without fertiliser and the larger leaves trimmed by half. The plants were covered with polythene to maintain high humidity whilst weaning. Gradually holes were slit in the polythene to decrease humidity before weaning was completed. One week after weaning, the small plants were infected with 200 hatched J2 *Meloidogyne javanica* nematodes. Watering with liquid feed thereafter was only done once the soil had dried sufficiently to cause the leaves to start wilting. The rootrainers were placed in trays on heated matting to maintain the soil temperature between 25-30°C. The leaves were trimmed back once a week in order to even out growth and prevent the growing points becoming covered with larger leaves due to density of planting.

The plants were harvested for scoring approximately 4 weeks after infection, and the roots were washed clean of soil and bleached for 4 minutes in 1% sodium hypochlorite. The bleach was removed by rinsing with water and then soaking in a large volume of water over 15 minutes with occasional agitation. The roots were then placed in 10 to 15 ml of a

1:500 dilution of acid fuchsin stock solution in 5% acetic acid. (Acid fuchsin stock was prepared according to 'Introduction to Plant Nematology' by V. H. Dropkin, ISBN 0-471-85268-6. Dissolve 0.35g acid fuchsin in 100 mls of 1:3 glacial acetic acid to distilled water). The samples in stain were placed in a boiling water bath for 4 minutes and transferred to 37°C for four hours. The stain was decanted and the samples are cleared by adding acidified glycerol and incubating at 37°C overnight.

The cleared roots with stained nematodes were then mounted in petri-dishes (the sample was placed on the inner side of the lid of a petri dish and the base of the petri dish is used to spread out and compress the sample for easier viewing under the microscope). The samples were viewed at 20 to 100 x magnification and nematodes were scored in several ways. Root knot nematodes were categorised into three groups: a) vermiform nematodes, b) saccate nematodes that are not producing eggs and c) saccate nematodes producing eggs. The diameters of the essentially saccate nematodes were measured using an eyepiece graticule.

The sizes of the infecting nematodes were significantly reduced in the transgenic Pro-PAP-S tobacco lines in comparison to those nematodes infecting control plants (Figure 13). This indicates that plants expressing Pro-PAP-S exert an inhibitory effect on nematode development.

Nematode resistance screening of potato transformed with PAP-S constructs

21 transgenic lines containing Pro-PAP-S were entered into a resistance trial with potato cyst nematodes, *Globodera pallida*, race 2/3. Control susceptible (Hermes) and partially resistant (Sante) varieties were included in the trial.

Primary resistance trials of transgenic plants were conducted in blind randomised trials on batches of 20 to 25 transgenic lines with at least ten replicates of 2 or more control lines. Rootrainers or conetainers were filled with a mixture of 50:50 loam and sand. 12 litres of loam and sand is moistened with 1250 mls of water to give a 40% water content. 3 cysts were placed onto the roots of each plantlet which was then inserted into a hole in the compost and the compost gently closed around the roots. The plants were weaned and thereafter, only watered once weekly with liquid feed or when the soil had dried sufficiently. Once the plants reached approx. 10 cm in height, the tips were trimmed off to even out growth. The plants were grown for approximately three months to permit the cysts to mature. The plants were then allowed to dry out for another month. Cysts were recovered from the plants by washing the soil and roots vigorously in a beaker of 250ml water. The soil was permitted to settle for a few minutes and the supernatant poured into a large filter funnel with a 32cm diameter Whatman No. 1 filter paper disc. The

supernatant was left to stand in the funnel for a minute and then the centre of the solution surface was touched with a drop of Hederol detergent to displace the material on the surface meniscus of the solution to the side of the filter. The base of the filter was then pierced to remove the remainder of the solution. The filter disc was removed and the number of cysts adhering to it is counted.

Plants were regarded as showing signs of resistance if they were infected with fewer cysts that susceptible control lines. (Figure 12). The population of transgenic plants as a whole had a significantly reduced PCN infection. At least 2 transgenic lines (namely pATC05502 - 6, 8 16) showed a level of resistance as good as the commercial resistant variety Sante.

A comparison of potato plant transformed with Pro PAP-S, an antisense KNT1 or Cowpea Trypsin Inhibitor (CpTI) under the control of a KNT1 promoter, shows Pro PAP-S to confer greater resistance than transgenic lines containing either antisense KNT1 or CpTI (Figure 15).

KNT1 is a gene substantially specifically induced in giant cells during nematode infection. It is believed that KNT1 is important in the process of infection and giant cell development. Introducing an antisense KNT1 sequence was believed to inhibit the nematode infection process.

Cowpea Trypsin Inhibitor (CpTI) is a protein which inhibits the digestive enzymes in the nematode, and its expression should prevent nematode feeding and development. It is not a cell-death system *per se* but a direct attack on the nematode.

The comparative data suggest transformation of plants with a construct containing Pro PAP-S to have greater effect on reducing nematode infection than lines containing either antisense KNT1 or CpTI.

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RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Agrobacterium tumefaciens C58 pBIN05101	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40870

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 20 March 1997 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd

Address: 23 St Machar Drive
Aberdeen Scotland
UK AB2 1RY

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 31 March 1997 Terence Davis

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

Advanced Technology (Cambridge) Ltd.
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Cambridge.
CB4 4WA

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: AS ABOVE Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40870 Date of the deposit or of the transfer: 20 March 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 26 March 1997 [2]. On that date, the said microorganism was [X] [3] viable [ ] [3] no longer viable	

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd

Address: 23 St Machar Drive
Aberdeen Scotland
UK AB2 1RY

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Terence Dando

Date: 31 March 1997

[4] Fill in if the information has been requested and if the results of the test were negative.